THE METHYL RED AND VOGES-PROSKAUER REACTIONS WITH SPECIAL REFERENCE TO ROUTINE WATER ANALYSIS

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Bacteriologic examination of water is an important part of the work of public health laboratories. It is essential that the method employed for these examinations be simple, easily applied to a large number of examinations, and that it distinguish factors of sanitary significance. This means that pathogenic bacteria must actually be isolated or that some condition, which is habitually associated with pathogenic bacteria be established. It must show actual danger to the health of those who drink the water, or a strong presumptive evidence of danger. The group of colon aerogenes organisms has been the indicator by which we have presumed the purity or pollution of water. If this group of organisms can be divided into at least two classes, one of which has a habitat in the intestinal tract of animals and the other in places not usually subject to animal pollution, then it is not proper to accept as an indicator of dangerous pollution all members of the colon aerogenes group.

In this laboratory we make a great many water analyses. We have used the methods of the "Standard Methods of Water Analysis of the American Public Health Association." A very high percentage of the water samples received have given positive tests for the colon group of bacteria. That is, lactose broth has been fermented with gas production, plates made from the gas tubes have shown acid colonies on litmus lactose agar, and acid colonies furnished from the plates have again produced gas from lactose broth. A good many of these samples did not show a high agar or gelatin count. We therefore for some time have been of the opinion that probably we were condemning water supplies as being polluted that should not have been. It is not to be inferred that we have applied the same standards to isolated wells and springs as to water supplies of villages and cities. It is hard to say just exactly why we have not, if the group of colon aerogenes organisms is accepted as an indicator of pollution. If, how-

Received for publication March 22, 1918.

ever, the two types of this group, methyl red positive and Voges-Proskauer positive organisms, represent, respectively, pollution of fecal and contamination of soil origin, then the permissible number of organisms present of the colon-aerogenes group will depend on the divisions into which they are classified. A much larger number of bacteria of soil origin can be permitted than those of fecal origin. Some of the recent work along this line gives proof of such a classification of the organisms of the colon aerogenes group of bacteria.

Rogers, Clark, and Lubs, and their associates have been able to show that bacteria belonging to the colon-aerogenes group can be classified with a few exceptions into two distinct classes, the high and low $CO_2:H_2$ ratio organisms.

Rogers, Clark and Davis[1] working with organisms obtained from milk say: "The carbon dioxid to hydrogen ratio which occurs with the greatest frequency is approximately 1:1. Plotted on the frequency basis this ratio stands apart from all higher cultures. All cultures giving the 1:1 ratio are distinguished from the high ratio cultures by the amount of gas formed under exact conditions. This was uniformly less than the amount produced under identical conditions by the high ratio cultures."

Rogers, Clark and Evans[2] in their two papers giving the results of their work with organisms isolated from cows' feces and grain give further proof of their classification of members of the colon-aerogenes group of bacteria and say: "The low ratio group evidently includes those members of the colon group usually designated as Bacillus coli communis and Bacillus coli communior, while in the high ratio group we may recognize Bacillus lactis aerogenes, Bacillus acidi lactici and possibly Bacillus coloacae."

Clark and Lubs[3] by the use of indicators have described a very simple test, which distinguishes between the high and low $CO_2:H_2$ ratio organisms. This test is applicable to routine water examinations.

Levine[4] was able to show a correlation between the methyl red and the Voges-Proskauer reactions. The organisms alkaline to methyl red gave a positive Voges-Proskauer reaction, and vice versa. He says that organisms which give a positive Voges-Proskauer test are seldom found in feces and that the Voges-Proskauer like the high ratio and alkalinity to methyl red is characteristic of nonfecal strains.

PROCEDURE

During the spring, summer and fall we receive a large number of water samples collected from private and public supplies. These samples are collected in special containers prepared in the laboratory for this purpose. They are shipped in cases especially constructed for the preservation of the sample while en route. For our routine examinations we have used the methods prescribed by the Standard Methods of Water Analysis of the Am. Pub. Health Assn. with the exception of the amounts of water with which the fermentation tubes are inoculated. Five 10 c c portions from each sample of water was inoculated into each of 5 fermentation tubes, containing approximately 40 c c of a 1% lactose broth in place of the 10 c c, 1 c c and 0.1 c c portions of the

[1] Jour. Infect. Dis., 1914, 14, p. 411.
[2] Ibid., 1914, 15, p. 99.
[3] Ibid., 1915, 17, p. 160.
[4] Ibid., 1916, 18, p. 358.

Standard Methods. All organisms have been required to satisfy the completed test of the Standard Methods and to be gram-negative before accepted as a member of the colon group.

In the preparation of the mediums used in our routine water examination as in all other procedures, we have followed the Standard Methods for Water Analysis. The peptone, glucose, phosphate mediums of Clark and Lubs[3] has been used for the methyl red and Voges-Proskauer reactions. Test tubes were filled with this medium and after inoculation were incubated at 37 C. for 3 days. At the end of the incubation period the culture was divided approximately in half, one half being used for the methyl red test and the other for the Voges-Proskauer reaction. The potassium hydroxid used in the Voges-Proskauer test was a 10% solution of Mercks' "Highest Purity." The methyl red was prepared by dissolving 1 gm. of Kahlbaum's methyl red in 1,000 c c of 95% alcohol. It was found that 0.2 c c of this solution was turned a distinct red in 100 c c of a $\frac{n}{100,000}$ solution of H_2SO_4. The neutral control remained yellow. The results of these tests are compared on 200 organisms isolated from water as described above.

RESULTS

Tables 1, 2 and 3 have been arranged to show the number of cultures isolated from each water sample, the reactions of each culture to methyl red and Voges-Proskauer tests, when grown in the dipotassium phosphate medium of Clark and Lubs, the intensity of these reactions, and the gas produced in 24 and 48 hours in pure and mixed culture. The gas was measured by noting the displacement of the medium down the closed arm of the fermentation tube, and is expressed in per cent. We realize the inaccuracies of this rough estimation, but believe for our purpose it is significant. We have used the average gas production by members of a type to indicate the influences of certain factors, growth in mixed and pure culture and incubation temperature, on the growth of members of that type.

It will be seen from Tables 1 and 2 that there was 120 of the 200 organisms which gave a positive methyl red test and 38 a positive Voges-Proskauer reaction, when first isolated. These organisms gave an undoubted reaction when first isolated, and reacted in the same manner to the second test. Table 3 shows the organisms which have reacted irregularly either by a double test or because in the second test the reaction was different from that obtained in the first test. In the first test there were 29 organisms which either gave a slight reaction to the methyl red and a comparatively strong reaction to the Voges-Proskauer tests or vice versa or a reaction of equal intensity to both tests. Because of these irregular reacting organisms, we decided to test all organisms again for the Voges-Proskauer and methyl red reactions, and gas production in pure culture.

TABLE 1

METHYL RED POSITIVE ORGANISMS

Water Sample	Culture Number	Gas in Lactose				Peptone Phosphate			
		Mixed (Hours)		Pure (Hours)		1st Test		2d Test	
		24	48	24	48	MR	VP	MR	VP
QA	132	30	40	5	10	4+	0	3+	0
	133	40	50	30	35	4+	0	4+	0
	134	40	40	35	35	4+	0	3+	0
	136	25	30	25	30	4+	0	4+	0
XA	162	10	40	0	20	4+	0	4+	0
	163	10	30	15	20	4+	0	2+	0
	164	15	35	0	20	4+	0	4+	0
	165	5	35	5	15	4+	0	4+	0
PB	178	35	40	30	35	+	0	4+	0
	185	15	20	30	35	+	0	4+	0
	186	40	60	10	20	+	0	4+	0
	189	35	60	10	15	+	0	4+	0
AB	175	60	90	35	40	4+	0	3+	0
	176	35	60	25	35	4+	0	3+	0
	177	40	90	25	30	4+	0	4+	0
	179	45	60	25	30	4+	0	4+	0
X	65	5	35	10	15	+	0	3+	0
	66	15	25	5	10	+	0	3+	0
	69	10	30	5	25	+	0	4+	0
YA	166	5	10	35	35	4+	0	4+	0
	167	15	30	25	30	4+	0	4+	0
	168	15	20	35	40	4+	0	4+	0
D	8	20	40	20	30	+	0	4+	0
	9	10	30	15	30	+	0	4+	0
E	11	10	15	5	15	+	0	4+	0
	12	25	30	5	10	+	0	4+	0
F	14	30	40	25	25	+	0	4+	0
	15	40	50	35	35	+	0	2+	0
G	16	5	5	5	5	+	0	4+	0
	17	10	10	5	10	+	0	4+	0
H	19	5	15	0	10	+	0	4+	0
	20	5	15	5	5	+	0	4+	0
J	25	40	40	25	35	+	0	3+	0
	26	30	30	30	35	+	0	4+	0
P	42	0	10	15	30	+	0	4+	0
	43	0	10	30	40	+	0	4+	0
LB	117	35	40	60	60	+	0	3+	0
	118	40	50	15	30	+	0	3+	0
PA	129	10	60	5	5	1+	0	2+	0
	130	5	60	5	5	4+	0	3+	0
UA	138	5	20	35	35	4+	0	3+	0
	139	0	5	0	0	4+	0	4+	0
MB	141	25	35	40	40	+	0	4+	0
	146	40	40	60	80	+	0	3+	0
A	1	10	20	30	30	+	0	4+	0
C	7	10	35	30	30	+	0	3+	0
K	27	15	20	15	20	+	0	4+	0
M	47	0	15	20	30	+	0	4+	0
W	63	5	50	20	30	+	0	4+	0
Z	74	30	30	20	20	+	0	4+	0
DA	86	0	40	20	20	+	0	4+	0
NA	102	10	10	20	25	+	0	3+	0
GA	103	10	20	15	20	+	0	4+	0
IB	106	5	30	25	30	4+	0	4+	0
MA	119	0	30	0	15	3+	0	3+	0
SA	150	0	40	5	10	4+	0	4+	0
EB	195	10	40	30	35	4+	0	4+	0
FB	196	25	40	40	40	4+	0	4+	0

Water Sample	Culture Number	Gas in Lactose				Peptone Phosphate			
		Mixed (Hours)		Pure (Hours)		1st Test		2d Test	
		24	48	24	48	MR	VP	MR	VP
N	36	20	20	20	25	+	0	4+	0
	37	30	40	20	25	+	0	4+	0
	38	10	15	25	30	+	0	4+	0
	39	30	30	30	30	+	0	4+	0
	40	20	20	30	40	+	0	4+	0
T	46	35	40	35	35	+	0	4+	0
	57	25	25	20	30	+	0	4+	0
	58	15	15	20	25	+	0	4+	0
	59	20	20	20	30	+	0	4+	0
	60	20	25	15	25	+	0	4+	0
ZA	170	40	40	35	40	3+	0	3+	0
	171	40	40	30	40	3+	0	4+	0
	172	40	40	40	40	3+	0	2+	0
	173	50	60	40	40	3+	0	2+	0
	174	40	60	40	40	2+	0	3+	0
BB	180	30	30	20	40	4+	0	4+	0
	181	30	35	25	40	4+	0	4+	0
	182	30	35	40	40	4+	0	4+	0
	183	40	40	40	40	4+	0	4+	0
	184	40	40	20	35	4+	0	4+	0
HB	6	10	15	30	40	+	0	4+	0
	121	20	35	20	30	3+	0	4+	0
	123	30	35	10	15	3+	0	4+	0
	135	25	30	40	40	4+	0	4+	0
	137	30	40	25	30	4+	0	3+	0
NB	147	35	40	50	60	+	0	4+	0
	148	20	30	50	50	+	0	4+	0
	149	30	40	50	50	+	0	4+	0
	151	25	35	70	70	+	0	4+	0
	155	25	35	50	50	+	0	4+	0
B	2	5	30	0	10	+	0	4+	0
	3	10	25	5	5	+	0	4+	0
	4	10	30	15	30	+	0	4+	0
	5	15	30	5	5	+	0	4+	0
DB	13	0	30	30	35	+	0	3+	0
	188	10	40	40	40	2+	0	4+	0
	190	35	60	10	15	+	0	4+	0
	191	25	35	25	30	4+	0	4+	0
GB	20	5	15	5	5	+	0	4+	0
	21	0	15	10	20	+	0	4+	0
	22	0	5	0	0	+	0	3+	0
	198	60	60	35	35	4+	0	4+	0
KB	23	50	50	40	40	+	0	4+	0
	87	50	50	40	40	+	0	4+	0
	88	40	50	15	20	+	0	4+	0
	96	40	60	60	60	+	0	4+	0
L	31	5	5	30	30	+	0	4+	0
	32	5	30	25	30	+	0	4+	0
	33	10	20	30	35	+	0	3+	0
	34	10	10	25	30	+	0	3+	0
S	51	25	25	15	20	+	0	3+	0
	53	40	40	30	35	+	0	4+	0
	54	20	20	35	40	+	0	4+	0
	55	20	25	15	30	+	0	4+	0
CA	89	10	40	5	10	+	0	4+	0
	90	15	25	20	25	+	0	4+	0
	91	15	25	25	40	+	0	4+	0
	92	5	20	5	25	+	0	4+	0
FA	97	0	40	20	20	4+	0	4+	0
	98	20	40	25	25	+	0	4+	0
	99	0	20	30	30	4+	0	4+	0
	100	0	30	10	20	4+	0	4+	0

A study of Tables 1 and 2 with reference to the gas produced after 24 hours in the mixed and pure cultures shows that in 29% of the methyl red positive organisms there was an increase of 20% or more in the gas formation in the second 24 hours' incubation in the mixed

TABLE 2

VOGES PROSKAUER POSITIVE ORGANISMS

Water Sample	Culture Number	Gas in Lactose: Mixed 24 Hours	Mixed 48 Hours	Pure 24 Hours	Pure 48 Hours	Peptone Phosphate: 1st Test MR	1st Test VP	2d Test MR	2d Test VP	Water Sample	Culture Number	Gas in Lactose: Mixed 24 Hours	Mixed 48 Hours	Pure 24 Hours	Pure 48 Hours	Peptone Phosphate: 1st Test MR	1st Test VP	2d Test MR	2d Test VP
AA	77	30	80	60	80	0	+	0	3+	EB	192	35	60	60	90	0	1+	0	2+
	78	5	50	20	60	0	+	0	4+		193	5	50	35	70	0	2+	0	1+
	79	40	90	60	80	0	+	0	2+		194	10	40	0	30	0	3+	0	2+
	80	30	50	40	60	0	+	0	1+	EA	29	20	70	35	80	0	+	0	2+
	81	10	40	20	60	0	+	0	3+		30	0	80	30	50	0	4+	0	3+
Z	72	50	50	80	90	0	+	0	3+	QB	46	60	90	50	60	0	4+	0	4+
	73	40	40	5	10	0	+	0	1+		48	10	80	40	40	0	4+	0	4+
	75	50	70	60	90	0	+	0	4+	R	49	10	10	35	60	0	+	0	2+
	76	40	65	60	90	0	+	0	3+		50	15	20	10	30	0	+	0	1+
OB	157	40	50	70	90	0	+	0	3+	Q	28	0	5	35	60	0	+	0	1+
	159	30	50	90	90	0	2+	0	3+	BA	35	10	50	40	70	0	+	0	3+
	160	25	40	90	90	0	+	0	4+	P	41	0	15	15	30	0	+	0	2+
	161	25	35	50	90	0	+	0	1+	S	52	25	35	35	40	0	+	0	2+
W	61	5	20	15	30	0	+	0	3+	X	67	15	15	20	40	0	+	0	2+
	62	0	40	5	10	0	+	0	1+	V	71	0	10	0	0	0	+	0	4+
	64	5	30	10	30	0	+	0	2+	Y	82	0	40	10	20	0	+	0	3+
HA	108	0	10	10	40	0	3+	0	3+	OA	124	80	90	20	30	0	3+	0	4+
	109	0	25	10	30	0	2+	0	2+	PA	131	5	50	5	35	0	3+	0	3+
	110	0	10	10	30	0	2+	0	4+	WA	158	5	5	5	35	0	1+	0	2+

TABLE 3

IRREGULAR REACTING ORGANISMS

Water Sample	Culture Number	Gas in Lactose: Mixed 24 Hours	Mixed 48 Hours	Pure 24 Hours	Pure 48 Hours	Peptone Phosphate: 1st Test MR	1st Test VP	2d Test MR	2d Test VP	Water Sample	Culture Number	Gas in Lactose: Mixed 24 Hours	Mixed 48 Hours	Pure 24 Hours	Pure 48 Hours	Peptone Phosphate: 1st Test MR	1st Test VP	2d Test MR	2d Test VP
JA	112	0	30	5	15	2+	2+	2+	3+	OA	125	60	70	40	60	1+	1+	3+	1+
	113	0	20	5	15	0	2+	1+	3+		126	10	60	10	20	1+	1+	2+	3+
	114	0	20	15	20	2+	1+	2+	2+	PA	127	60	70	5	20	1+	2+	2+	0
	115	0	20	5	30	2+	1+	2+	1+		128	60	70	0	5	3+	2+	2+	4+
	116	0	15	10	40	2+	1+	2+	1+	U	70	0	10	5	20	+	+	3+	1+
TA	142	0	30	0	10	0	3+	1+	3+	Y	83	0	20	5	15	+	+	3+	3+
	143	0	30	5	40	1+	2+	2+	3+	KA	94	0	30	40	40	+	+	2+	1+
	144	0	25	5	30	2+	2+	2+	2+	MA	120	0	40	5	40	2+	1+	2+	4+
	145	5	10	5	50	1+	3+	1+	2+	YA	169	15	25	10	35	1+	1+	2+	1+
VA	152	5	35	10	40	0	2+	2+	3+	E	10	0	5	20	30	+	+	0	1+
	153	10	45	5	50	0	4+	3+	3+	G	18	0	15	40	60	+	+	0	1+
	154	10	20	0	30	0	1+	2+	2+	I	24	0	5	15	30	+	+	0	1+
	156	5	15	0	30	1+	3+	2+	3+	UA	140	0	10	5	35	1+	3+	0	2+
GA	104	0	20	5	60	0	2+	1+	3+	LA	95	0	30	20	40	+	+	0	2+
	105	0	25	5	40	1+	1+	1+	4+	CB	187	0	30	0	0	1+	1+	3+	0
	107	0	20	5	15	1+	2+	3+	0	FB	197	5	40	35	40	2+	1+	4+	0
O	44	0	10	10	30	+	+	3+	1+	JB	111	40	60	0	0	0	2+	2+	3+
	45	0	10	15	30	+	+	2+	0	X	68	5	15	0	0	0	+	4+	0
IA	84	0	10	5	10	0	+	1+	3+	CA	93	5	30	0	0	0	0	1+	0
	85	0	35	5	10	+	+	1+	4+	GB	199	40	50	25	90	0	3+	0	0
NA	101	10	30	0	0	+	2+	4+	0										
	122	0	10	40	50	2+	1+	2+	1+										

cultures, and that in the pure cultures only 4% have shown as much as 20% increase in gas after the first 24 hours.

In the group of Voges-Proskauer positive organisms a little more than 57% had an increase of 20% or more of gas in the second 24 hours, in the mixed culture, and 66% shows a similar increase when grown in pure culture.

In pointing out in the foregoing the effects of growth in a mixed culture on the growth of the different groups of organisms as indicated by gas formation, individual cultures, without reference to the samples of water from which they came, have been used. Tables 1, 2 and 3 indicate that some samples of water yielded organisms belonging to the same group from all five of the portions planted, while others yielded organisms of two or more types. This is conveniently shown in the summary which follows.

From 28 waters all cultures isolated were M. R. +.
From 10 waters all cultures isolated were V. P. +.
From 19 waters some cultures isolated were M. R. + and some V. P. + and some irregular.
From 11 waters all cultures gave an irregular reaction.

It is therefore reasonable to conclude that in certain (28) samples of water only colon organisms of the methyl red group were present, and in certain other samples (10) only colon organisms of the Voges-Proskauer group were present. If, now, we consider only cultures which came from water samples from which all cultures isolated had a like reaction, we find in the average gas production in the first 24 hours in mixed and pure culture quite a difference. It is seen then that there are 10 water samples all cultures from which were positive to the Voges-Proskauer reaction. These cultures gave an average gas formation of 17% in the first 24 hours in the mixed culture, and 37% in the same period when growing in pure culture. The methyl red cultures selected and compared in the same manner show no difference in the gas formation in the mixed and pure cultures. These results are shown in Table 4.

At the same time the fermentation tubes were inoculated with portions from the water samples for examination, agar and gelatin plates were also made. We thought that possibly there might be some correlation between the agar and gelatin counts and the reaction of the organisms isolated. Table 5 shows the results of agar and gelatin counts in water samples from which all cultures from the sample reacted alike.

We realize that there are too few water samples reported in this series from which to draw any absolute conclusion. It is seen, however, that 50% of the samples giving only Voges-Proskauer positive organisms give a high gelatin count and 30% a high agar count, and 30% high in both. Those samples from which organisms of the methyl red group only were obtained show a high gelatin count in 48+%, high agar count in 44+%, and high in both in 40+%. It must be borne in mind that some of these water samples were collected

TABLE 4

SHOWING THE AVERAGE GAS FORMATION IN THE FIRST 24 HOURS' INCUBATION OF THE METHYL RED POSITIVE AND VOGES-PROSKAUER POSITIVE ORGANISMS IN MIXED AND PURE CULTURE

	Water Samples	Culture Isolated	Gas Percent Culture	
			Mixed	Pure
V. P. +	10	22	17	37
M. R. +	28	85	25	25

from farm wells, and that in most cases they were exposed to gross pollution, which, of course, included many soil bacteria as well as bacteria of fecal origin.

In the first set of tests we found 29 organisms which gave an irregular reaction. We at first decided to do a duplicate test on these and then later decided to do a duplicate test on all of the cultures. The first set of tests therefore represent the reactions of the organisms

TABLE 5

SHOWING THE RELATION OF THE AGAR AND GELATIN COUNTS OF WATERS YIELDING ONLY ONE TYPE OF ORGANISMS

Organism Type	Number Waters	High Gelatin Count (1,000 or Above per C C)	High Agar Count (100 or Above per C C)	High in Both	Low in Both (Below 1,000 on Gelatin; Below 100 on Agar per C C)
V. P. +	10	5	3	3	5
M. R. +	28	13	12	11	14

when they were first isolated, and the second set the reactions about 6 months later. We soon learned that some organisms would apparently give a double positive reaction, that is, both a Voges-Proskauer and a methyl red. In some cases one would be much stronger than the other so that if we had classified them according to the intensity of the color reaction many would have been included in our groups of methyl red and Voges-Proskauer positive organisms which have not been. Some of the reactions gave a double positive reaction of

equal intensity, altho such reactions were usually weak in both cases, as is shown in Table 3. Because of this variation in intensity we marked the reactions as 1+, 2+, 3+, and 4+, according to whether the color was slightly red, red, a deep red, or a very deep red, and similarly with the pink in the Voges-Proskauer reaction.

Table 3 shows the results of the test of the irregular organisms. We have placed in this class every organism which has not reacted positive to either one test or the other and so reacted throughout both tests. We therefore have in this group all organisms which gave a double reaction regardless of the intensity of the reaction, and all which in any way had changed reaction in the second test. This makes the group of irregular reactors seem rather high. We are sure, however, that we have no organisms in either of the positive groups which do not belong there.

Table 3 is arranged to show all of the reactions of these organisms. It will be seen that 20 gave a double positive reaction in both tests, that 5 changed from a double positive to a Voges-Proskauer positive, that 6 changed from a double positive to a methyl red positive, that 8 changed from a Voges-Proskauer positive to a double reaction, and that Nos. 68, 93, and 199 were irregular to one or more of the reactions. Of the 25 waters, which furnished the 42 irregular cultures, 14 furnished one or more methyl red positive or Voges-Proskauer positive, or Voges-Proskauer and methyl red positive organisms along with the irregular reactors. Eleven furnished only irregular organisms. One water sample furnished an irregular reactor from each of the 5 fermentation tubes inoculated and 2 samples four each from the 5 tubes planted.

We hope to report soon on the classification of these irregular reactors by accurate gas analysis.

DISCUSSION

The influence of growth in mixed cultures on the isolation from water of organisms of the methyl red and Voges-Proskauer positive types in the colon group is of particular interest in the routine examination of water. It has been shown by Clark and Roger and others that in pure culture under anaerobic conditions the methyl red positive organisms produce a smaller amount of gas than the Voges-Proskauer, and further that the methyl red positive type reach their limit of H-ion concentration and stop growing, while the Voges-Proskauer type may form acid to a certain H-ion concentration, and

then certain other reactions intervene to reduce the H-ion concentration formed and this process starts over.

In the fermentation tube which has growing in it many types of bacteria, which may be found in almost any sample of water, conditions are entirely different and the reactions therefore must be different. In those tubes which have been inoculated with portions from water samples, both methyl red positive, and Voges-Proskauer positive organisms, not to mention the variety of other bacteria, may be growing. A study of the gas produced in Tables 1 and 2 we think throws some light on how these two types react in mixed and pure culture. We have not compared the volume of gas produced by each individual culture because we were aware of the errors into which we would fall. We have, however, used the average gas production of the whole group for the periods which we wished to compare, and believe that the results indicate what has actually happened.

We find that by comparing the gas produced in the first 24 hours in fermentation tubes from which methyl red positive organisms were isolated with the average amount of gas produced in the second 24 hours' incubation, that 29% show an increase of 20% or more in gas produced. A similar comparison made after the culture was isolated and growing in pure culture shows that only 4% of the cultures show such an increase. However, the average amount of gas formed (25%) in tubes which were inoculated from samples of water from which only methyl red positive organisms were isolated during the first 24 hours' incubation was exactly the same as that formed by these organisms when growing in pure culture. We believe, therefore, that other reactions brought about by other organisms reduced or partially reduced the H-ion concentration until the organisms isolated established its predominance in the second 24 hour period of incubation. It appears, therefore, that the methyl red positive organisms growing in pure culture may not establish their predominance until after 24 hours' incubation at 37 C. It appears that when Endo plates made from tubes showing gas in the first 24 hours fail to show characteristic colon colonies, plates made at the end of 48 hours may show such colonies in numbers.

In the case of the Voges-Proskauer positive types found in our group a marked difference in the average amount of gas produced in the pure and mixed culture, and in the average increase in the second 24 hours was found. In this group 57% growing in the mixed

culture, and 66% growing in pure culture showed 20% or more increase in gas produced in the second 24 hours.

When we examine the average gas production in tubes inoculated from water samples from which only Voges-Proskauer organisms were isolated during the first 24 hours in the mixed and pure culture, a marked difference is noted. The average gas production in the mixed culture was only 17% while in the pure culture it was 37%. When we put these figures together we find that a considerably smaller number gave as much as a 20% increase in gas formation during the second 24 hours in the mixed culture, and also that the average gas production was considerably less in the first 24 hours. This indicates that the organisms of this type have been inhibited to a considerable degree from some cause. We believe that it may be the combination of the effect of growth in the same tube with many other organisms and the temperature, 37 C., of incubation. It is probable that the Voges-Proskauer positive class of the colon aerogenes group being of a nonfecal type do not find so high a temperature advantageous for their growth. On the other hand, the methyl red class being a fecal type would find such a temperature highly satisfactory for their development.

It is probable, therefore, that under the condition in which our routine water analysis is done we are favoring the growth of methyl red positive class of organisms, and inhibiting the growth of the Voges-Proskauer. It appears that the methyl red positive division is not inhibited to any degree by growing in mixed culture at 37 C., while the Voges-Proskauer are. This may be interpreted to mean that the methyl red positive division being direct pollution from the intestinal tracts of animals finds this temperature most favorable, while the other division, commonly soil bacteria, finds it unfavorable.

SUMMARY

In the routine analysis of 68 water samples from private and public supplies received from many sections of the state we have isolated 200 cultures which fulfilled the requirements of the completed test for members of the colon group of organisms of the Standard Methods of Water Analysis. We found that 120 of these reacted acid to methyl red, 38 gave a Voges-Proskauer positive reaction, and that 42 gave irregular reactions.

The intensity of these reactions varied considerably. In instances in which the same organism apparently gave both reactions positive it

was not always possible by the intensity of the reaction to say in what division it should be classed. In other instances, if the more intense reaction had been accepted as indicating the division to which the organism belonged, our group of irregular reacting organisms would have been much smaller.

It seems probable that the class of methyl red positive organisms is not interfered with in its development when growing with other bacteria found in water incubated at 37 C. as are the Voges-Proskauer. It is probable that in the same culture the methyl red positive will overgrow the Voges-Proskauer positive organisms. It seems advisable therefore whenever these reactions are used in water analysis to make Endo or litmus-lactose agar streak plates from the fermentation tubes at the end of 24 and 48 hours.

We are not able in the small number of water samples here reported to find any correlation between the agar and gelatin count and the type of organisms of the colon group isolated from the samples of water.